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(54) Title: METHOD AND MATERIALS FOR THE IDENTIFICATION OF LIPOPOLYSACCHARIDE PRODUCING MICROORGANISMS (57) Abstract Means for the immunological detection of an entire class of microorganisms in clinical samples. The detection is accomplished by reaction of the clinical sample with a class-specific immunological reagent. This reagent is an antiserum either monoclonal or polyclonal in nature, and the detection is based upon reaction of the antiserum with an antigenic determinant which is shared among all members of the detectable class of microorganisms. The presence of the resulting immunological reaction product (e.g. the antigen-antibody complex) may be detected by well-known immunological detection-systems.		

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1 METHOD AND MATERIALS FOR THE IDENTIFICATION OF
 LIPOPOLYSACCHARIDE PRODUCING MICROORGANISMS

Field of the Invention

 This invention relates to the immunological
5 detection of microorganisms. More specifically, the
 invention relates to methods and materials useful for the
 detection of a diverse group of microorganisms in a clinical
 sample.

Background of the Invention

10 Microorganisms possessing a cell-surface component
 known as an endotoxic lipopolysaccharide (LPS) have been
 identified as the etiologic agents in a wide variety of
 diseases. Of particular interest are strains of the genera
 Neisseria, and Chlamydia, which are associated with certain
15 venereal diseases; the incidence of these diseases having
 reached almost epidemic proportions.

 The LPS molecule has been the subject of intense
 study. See for example, Westphal et al., Bacterial
 Lipopolysaccharides, Methods of Carbohydrate Chemistry, Vol.
20 V, Academic Press, (1965), pgs. 83-91; and Galanos et. al.,
 Biochemistry of Lipids II, T.W. Goodwin (Ed), Univ. Park
 Press, (1977), Vol. 14, at page 239.

 The structure of the lipopolysaccharide has been
 described in studies of Gram-negative bacteria such as,
25 Escherichia coli and Salmonella typhimurium. In general, the
 antigen can be visualized as possessing three component
 regions. Proximal to and imbedded into the outer portion of
 the cell membrane is the so-called lipid A component. This
 component has been associated with the endotoxin properties
30 of the molecule and is believed to be a highly conserved
 sequence; that is to say, a wide variety of organisms would



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1 likely possess identical lipid A regions. The middle region
of the LPS molecule is another conserved region, the
so-called core oligosaccharide. The core oligosaccharide is
bipartite in structure based upon the types of sugar moieties
5 of which it is composed. The innermost region which is
adjacent to the lipid A component contains the unusual sugar,
3-deoxy-manno-octulosonic acid, also known as
ketodeoxyoctanoic acid (KDO). The outermost region of the
core structure is comprised of a region of seven-carbon
10 sugars (heptoses) which is followed distally by a region
containing six-carbon sugars (hexoses). The most distal
region of the LPS molecule is the so-called somatic or (O)
region comprised of highly-variable polysaccharide
components.

15 It is this outermost region which induces the
strongest immune response in infected organisms and it is to
this region that antisera produced thereby is directed.
Thus, even though a number of different taxa may possess the
same Lipid A and core components of the LPS molecules,
20 because of the antigenic variability of the somatic region,
antisera produced thereto will be specific for each type of
inducing organism.

Microorganisms can thus be classified into
chemotypes based upon their carbohydrate composition in the O
25 region of the LPS. One method is described by Merrick in U.S.
Patent 3,891,508. Such a composition analysis although
somewhat useful for purposes of identification is limited by
not being able to discriminate between two organisms which
may possess the same kinds of sugars but in different
30 proportions or in different sequences.



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1 The LPS molecule when used as an immunogen, may
produce certain toxic side-effects due to the biological
activity of the molecule. Several patents, such as U.S.
Patents 4,185,090 and 4,057,685 relate to methods of reducing
5 the toxicity of the molecule but retaining the antigenicity
of the O region. The specificity of the O region in
permitting the identification, of only a single species is
disclosed by Wallace et. al. in U.S. Patent 4,115,543
relating to the identification of Neisseria gonorrhoeae.
10 Other approaches attempt to find other antigenic inducers and
eliminate the LPS molecule from the immunizing mixture as for
example, described by Ayme in U.S. Patent 4,337,243.

It has been recently demonstrated that conventional
polyclonal sera raised to Salmonella typhimurium Re mutants
15 cross-react with various Chlamydia species (Nurminen, et al.,
Science 220: 1279-81 (1983)). There is no suggestion of the
diagnostic utility nor of the wider reactive potential of the
antibodies as produced and described hereinbelow.

Human monoclonals of the IgG₁ subclass have been
20 demonstrated to be reactive with a limited class of
microorganisms namely members of the Chlamydia genera.
Although, not particularly well-characterized, the antigen to
which the monoclonals are directed is referred to as a
lipoprotein complex. Thus, it is probably closely related to
25 the Chlamydia group specific protein as characterized by
Caldwell, et al., (J. Immuno. 115: 963-68 (1975)) and clearly
distinct from the endotoxic-glycolipid as described herein.

In an interesting, but unrelated application, (PCT
application WO80/01109) the LPS molecule itself has been used
30 as a marker reagent; being chemically attached to a ligand,



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- 1 it permits the detection of the LPS-ligand complex by
standard LPS assay method (The Limulus amoebocyte assay).

None of the above references relate to the object
of the subject invention, that being, the generation of
5 immunological reagents useful in the identification of a
diverse group of LPS producing microorganisms.

Brief Description of the Invention

The subject invention provides a means for the
immunological detection of an entire class of microorganisms
10 in clinical samples. The detection is accomplished by
reaction of the clinical sample with a class-specific
immunological reagent. This reagent is an antiserum either
monoclonal or polyclonal in nature, and the detection is
based upon reaction of the antiserum with an antigenic
15 determinant which is shared among all members of the
detectable class of microorganisms. The presence of the
resulting immunological reaction product (e.g. the
antigen-antibody complex) may be detected by well-known
immunological detection systems employing analytically
20 indicatable reagents.

The subject invention is particularly useful for
the detection of a class of microorganisms which produce
endotoxin or endotoxin-like molecules. The shared antigen
determinant in this case, is often a component of a
25 cell-surface lipopolysaccharide. Microorganisms which
display this antigen and thus are members of a detectable
group comprise Chlamydia, Rickettsia, and other gram-
negative microorganisms such as Neisseria, Brucella,
Escherichia, Salmonella and the like.

30 In a preferred embodiment, an antibody to the
shared determinant of the detectable class is used as the
"capture" antibody in a differential screening assay system.



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- 1 Once recovered from a clinical sample as a member of the
reactive class of microbes which possess the shared antigen,
the captured cells or antigens are then subsequently reacted
with more specific antisera to indicate which particular
5 genera, species or serotype of microorganism is present.

For example, a first antibody is synthesized which
reacts with the "gram-negative-like" portion of the
endotoxic glycolipid, and second antibody is synthesized
which reacts with a Chlamydial genus specific determinant.

- 10 The first antibody can be used alone to detect all gram-
negative-like microorganism by simple fluorescent or
agglutination assays. Alternatively, the two antibodies may
be used in combination in a solid phase immunoassay to
discriminate the Chlamydia genera from among the other
15 members of the reactive class.

- In a further embodiment, the Chlamydia genus
specific antibody may be used alone to detect all members of
the Chlamydia genus or as the capture antibody in a
differential screening assay whereby the Chlamydia genus
20 specific antibody is used to react with all members of the
genus and a second antibody or antibodies which are reactive
to Chlamydia species determinants are employed to
differentiate each species in the reactive genera..

- The subject invention provides an immunological
25 reagent for the detection of shared antigenic determinants of
at least two microorganisms comprising an antibody
specifically reactive to said shared determinants, more
specifically an immunological reagent for the detection of
two or more endotoxin or endotoxin-like lipopolysaccharide
30 producing microorganisms comprising a monoclonal antibody
specifically reactive with shared antigenic determinants of
said lipopolysaccharide.



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1 Also provided are methods for the immunological
detection of an antigen shared by a plurality of
taxonomically distinct microorganisms. One such method
comprises: contacting a sample containing said
5 microorganisms or antigens thereof with an analytically
indicatable antibody to form an immunocomplex of said
microorganism or antigen thereof and said antibody, said
antibody being characterized as having affinity for an
antigenic determinant shared by said microorganism. There is
10 also provided a two-site immunometric assay to identify an
antigen in a sample comprising forming a ternary complex of a
first labelled antibody, said antigen, and a second antibody,
said second antibody being bound to a solid carrier insoluble
under ternary complex forming conditions wherein the presence
15 of the antigen is detected and identified by measuring either
the amount of labelled antibody bound to said solid carrier
or the amount of unreacted labelled antibody, in which the
improvement comprises employing as said second antibody a
monoclonal antibody characterized in having affinity for an
20 antigenic determinant site of said antigen which is shared by
a plurality of taxonomically distinct microorganisms and
employing as a first antibody an antibody characterized as
having an affinity for an antigenic determinant site specific
to a taxonomically distinct microorganism.

25 Detailed Description of the Invention

This invention provides reagents and methods for
the detection and subsequent differential identification of
an entire class of microbes. For the purposes of this
description, class is not used in its strict
30 biological/taxonomic meaning but rather in a more general
sense referring to a group of microorganisms that express a



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1 common antigenic determinant. The terms antigenic
determinant, antigenic determinant site (ADS) or epitope as
used throughout, refer to a limited, specific part of an
antigen which is the inducer of antibody formation and which
5 is the region of the antigen to which the induced antibody
reacts. The number of antigenic determinant sites possessed
by a given antigen will vary and may range from 1 to 10 or 20
or more depending upon the size and immunological complexity
of the antigen molecule. Those antigenic determinants which
10 are unique to a given antigen are known as specific
determinants and are useful in discriminating between two
different types of antigen molecules. Those determinants
which are present on two or more antigens are known as shared
determinants and, as mentioned above, form the basis of an
15 immunological class of antigens.

An antigen of particular interest with respect to
the subject invention, as described above, is the lipopoly-
saccharide (LPS)/glycolipid antigen associated with endotoxin
or endotoxin-like molecules produced by Gram-negative
20 microorganisms.

With reference to the discussion of antigenic
determinants above, each of the three regions of the
LPS/glycolipid molecule could be considered to be comprised
of one or more separate ADSs. The somatic region is
25 considered to be a specific determinant since, because of its
high variability, antibodies raised to the somatic region of
one LPS would not likely cross-react with another O
determinant; thus organisms expressing a particular LPS may
be distinguished from others by virtue of the somatic
30 ("body") types.



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1 In contrast, because of the conserved nature of the
Lipid A or core region, different organisms that possess
endotoxin or endotoxin-like entities will have
antigenetically similar regions. Thus, antibodies raised to
5 these determinants will react with an entire class (group) of
microorganisms, those of which can express LPS regardless of
somatic type.

In Nature, the induction of anti-Lipid A or
anti-core poly-saccharide reactive antibodies does not occur
10 readily because of the existence of the somatic determinants
attached thereto. However, there are known to exist in
Salmonella mutations which affect various steps in the
synthesis of the LPS/glycolipid molecule. Certain of these
mutations result in strains which produce truncated forms of
15 the LPS/glycolipid molecule and are known as R mutants. One
of the most defective R types known is designated as Re.
This mutant is well-known having been described by Stocker,
et al. (J. Gen'l. Microbiol., 70: 527 (1972)) and is
publically available from the Salmonella Stock Center, Dept.
20 of Biology, University of Calgary, Calgary, Alberta, Canada.

As described herein it is possible to prepare
antibodies which react specifically with the Lipid A or
core-determinants thus providing valuable immunological
reagents for the detection of a class of microorganism, i.e.,
25 those microorganisms expressing LPS/glycolipid regardless of
somatic type.

A variety of protocols may be used to provide the
Lipid A or core-polysaccharide reactive antibodies.
Conventional immunization and isolation procedures result in

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- 1 the production and recovery of polyclonal antisera directed against the shared determinants of the LPS antigen. Alternatively, somatic cell hybridization procedures may be employed to construct hybridoma cell lines capable of
5 generating monoclonal antibody to the determinants in question. These procedures are more fully described in the Examples which follow.

Although it has been recently demonstrated that conventional polyclonal sera raised to Salmonella typhimurium
10 Re mutants cross-react with various Chlamydia species, (Nurminen, et al., Science 220: 1279-81 (1983)), there is no suggestion of the diagnostic utility nor of the wider reactive potential of the antibodies as produced and described herein.

- 15 It has been surprisingly discovered that the immunological reagents described herein not only react with Gram-negative bacteria expressing LPS such as Salmonella and Neisseria, but also all Chlamydia species as well. Predicated on this finding it is now possible to generate
20 immunological reagents of extremely broad specificity such as a single antibody reactive with any and all LPS/glycolipid producing organism. Appropriate target organisms include Gram-reactive bacteria such as Salmonella, Brucella, Escherichia, Neisseria, Serratia, Pasteurella, Proteus,
25 Shigella, Klebsiella, as well as Chlamydia, and Rickettsia.

- Furthermore a group of immunological reagents of intermediate specificity are also disclosed herein. Antibodies of the group react specifically with members of all species within a given genera but not with organisms
30 outside that genera. These antibodies are said to be genera-specific.



1 Thus, by using an antibody of broad specificity, it
is possible to perform a single test for a large number of
LPS producing organisms merely by reacting said antibody with
a clinical sample and detecting any antibody-antigen reaction
5 which occurred by measuring the antigen and antibody complex
directly, conventionally by monitoring an analytically
indicatable detection system.

 Thus antibodies of the subject invention can be
used directly and can be modified as necessary to render them
10 useful in any of the well-known immunological detection
systems. Although not meant to be exhaustive, suitable
methods for detection of the antigen-antibody reaction
include: precipitation, agglutination, double antibody
techniques, fluorescent-, enzyme-, ferritin-, or
15 radioactively-labelled antibodies ¹²⁵I-/or similarly
radioactively labelled antibodies, protein A from
Staphylococcus aureus, enzyme-linked immunoassay (ELISA) or
biotin-based assay systems. The subject invention provides
specific reagents for the reaction with the LPS/glycolipid
20 antigen and not any particular method of indicating the
resulting reaction product. The most appropriate method of
indication can be determined by one skilled in the art. A
wide variety of indicator systems are contemplated, and the
subject invention should not necessarily be limited by a
25 particular method of indicating the positive reaction.

 In a preferred embodiment, the immunological
reagent (the anti-Lipid or anti-core LPS reactive antibody of
or monoclonal origin) is employed as a capture antibody in a
"sandwich-type" assay as described in U.S. Patent 4,376,110.
30 According to this embodiment, the anti-Lipid A or



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- 1 core-reactive antibody is attached to a solid support by widely known cross-linking methods such as described by S. Avrameas In: Immunoassays for the 80's Voller et al. (Eds) University Park Press, Baltimore (1981) at page 85.
- 5 A clinical sample, which may be defined as body fluids or secretions such as blood, serum, saliva, stool, urine, milk, topical washing of skin or genitals, tissue samples or homogenates thereof and samples of culture fluids of infected cells or homogenates thereof, is reacted with the
- 10 supported immunological reagent under conditions which promote the formation of an immune complex between said supported reagent and any material containing the Lipid A or core determinants, thereby binding to the support one or more members of a variety of taxonomic groups of micro-organisms.
- 15 The specific identity of the constituent members of the bound population may be determined, after washing the initial reaction product to remove unbound contaminants, by subsequently reacting the bound population with specific antisera for each specific taxonomic type of interest e.g.
- 20 genera-specific, species-specific or serotype-specific antisera. An advantage employing a reagent of broad specificity is that considerable research and development as well as production time and expense is saved by eliminating the necessity of generating a large number of reagents of
- 25 narrower specificity to accomplish the same result.

In a further embodiment, the above described "sandwich" assay can be carried out by providing a plurality of separate reactions of the supported "capture" reagent followed by detection of the reacted sample by adding a

30 particular genera-, species- or serotype-specific reagent to



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- 1 an individual reaction or alternatively two or more specific reagents may be added to a single reaction. Thus it is possible to detect simultaneously in one reaction two or more taxonomically distinct microorganisms. In this later
- 5 variation of the assay, if the clinical sample contains both Chlamydia as well as Neisseria both organisms would be retrieved by the "capture" antibody attached to the solid support. Once bound, addition of two additional types of detectably labelled antibodies, one specific for Chlamydia
- 10 and the other specific for Neisseria would permit the simultaneous detection of both organisms. Detection is facilitated by employing analytically distinct reagents is labelling the specific antibody. For example, the Chlamydia specific antibody may be labelled with B-galactosidase and
- 15 the product of the ELISA reaction measure of one spectrophotometric wavelength whereas the Neisseria specific antibody labelled with alkaline phosphatase and the ELISA reaction product measured at a second wavelength. Other variations employ colorimetric blending of two separate
20. reaction products or the use of a radiometric/photospectrometric combination are also possible. The following examples further illustrate various facets of the invention but are not to be viewed as limitations of the invention per se.



Example 1

New Zealand white rabbits weighing approximately 2

Other rabbits and mice were immunized in a similar

30



1 with acetic acid. This lipid A was adsorbed to the acetic
acid-treated whole bacteria by mixing into a homogeneous
suspension the lipid A and the acid-treated bacteria and then
evaporating the water under reduced pressure at 50-60°C until
5 a thick slurry was generated.

Animals immunized with this antigenic preparation
produced a sera termed anti-lipid A glycolipid antisera. In
certain circumstances, some of the animals received an equal
mixture of untreated whole cells and acid-treated, lipid
10 A-coated whole cells. Sera from these mice is termed
anti-Re-Lipid A glycolipid antisera.

These antisera were tested in an enzyme immunoassay
for reactivity to purified endotoxic glycolipids (termed Re
endotoxin or lipid A) prepared as described above. These
15 glycolipids were attached to polystyrene plates by incubating
0.1 ml/well of a solution containing endotoxic glycolipid (25
ug/ml), triethylamine (0.5 ul/ml), sodium azide (0.15) and
MgCl₂ (1 mM) for 6 to 18 hours at 37°C. This solution was
removed and replaced with phosphate buffered saline (pH 7.4)
20 containing 1 mM MgCl. The plates were stored at 4°C. The
test antisera or normal rabbit sera or normal mouse sera at
various dilutions (commonly 1/1000) was added to each well
(100 ul/well after removal of the saline solution used for
storage). The plates were incubated 3 to 18 hours at 25 or
25 37°C; the sera was decanted; the plates were washed with
buffered saline containing MgCl (three times, 200 ul each
time); alkaline phosphatase-conjugated antiserum to rabbit or
mouse immunoglobulin (IgG and IgM) (purchased from Tago Inc.,
Burlingame, CA) was added and incubated for 6 to 18 hours at
30 37°C. The plates were again washed as before and 100 ul of



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- 1 alkaline phosphatase substrate p-nitro-phenol phosphate in 1% diethylamine and 1 mM MgCl was added. The plates were examined visually for yellow color development or spectrophotometrically at 405 nm following incubation at 25°C
- 5 for 1/2-3 hours.



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Example 2Antibody to Endotoxins of ChlamydiaSpecies and Neisseria Species

Rabbits and mice were immunized with formalin-fixed
5 elementary bodies of C. trachomatis (strain L2/434/Bu(L2)
grown in HeLa cells (see Caldwell et al. Infect. Immun. 31,
1161 (1981) for preparation of purified elementary bodies).
The formalin fixation and immunization scheme was as
described in Example 1 above. Other animals were immunized
10 with formalin-fixed N. gonorrhea (laboratory strain F62
phenotyped $P^{++}O^{+}$) in a similar immunization protocol.

Sera from these animals was tested for antibody
reactivity in an immunoassay as described above in Example
1. Whole elementary bodies or endotoxic extracts C.
15 trachomatis or whole bacteria N. gonorrhea were attached to
polystyrene plates (10^7 organisms or equivalent extract per
ml of phosphate buffered saline; 0.15 ml per well). The
endotoxic extracts were obtained as described in Example 1.
The plates were subsequently treated with MEM solution
20 containing 10% fetal calf serum and 0.25% bovine serum
albumin (0.25 ml per well). Some of the wells were treated
with sodium metaperiodate (50 mM) in sodium acetate (50 mM,
pH 5.6 for 3 hours at 37°C. This treatment destroys the
immunological reactivity of certain sugars in the endotoxins
25 or endotoxic glycolipids, but does not affect the
immunological reactivity of peptides in proteins.



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Example 3Monoclonal Antibody to Endotoxic Glycolipids

A. Female BALB/c mice were immunized with a mixture of untreated and acid-treated cells as described in Example 1. The mice received 4 to 6 injections of immunogen and were euthanized three days after the last injection. The spleens were removed aseptically and homogenized between glass microscope slides. The cells were washed in minimum essential medium (MEM) and the red blood cells lysed by treatment for 45 seconds in sterile water. The cells were diluted into MEM to stop the lytic process. The spleen cells remaining were pelleted at room temperature upon centrifugation for 15 minutes at 1500 rpm in a Beckman TJ-6 table top centrifuge. To this pellet was added 2 to 5 x 10⁷ SP2/0 myeloma cells (Institute for Medical Research, Camden, N.J. 08103) that had been washed twice in MEM. Typically spleen cells from one spleen were combined with myeloma cells recovered from vigorously shaking three, 250 cm² Falcon tissue culture flasks used for maintenance of the myeloma cell line.

The combined spleen cells and myeloma cells were centrifuged for 10 minutes at 600 rpm in the Beckman TJ-6 centrifuge at room temperature. The pellet was dislodged after decanting the MEM. The cells were then treated for 1 minute with 1.0 ml of polyethylene glycol 4000 (Sigma Chemical Co.) which had been sterilized and diluted with an equal part of MEM just before use. It was important to dilute the polyethylene glycol with MEM while it was still hot from sterilization. It was cooled to 37°C. before addition to the spleen cell and myeloma cell pellet. Sequential additions of 1.0, 2.0, and 4.0 ml of MEM followed



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- 1 at 1.0, 2.0, and 4.0 minute intervals. The fusion treatment was terminated by the addition of 43 ml of MEM with 10% fetal calf serum (Hyclone Laboratories, Sterile Systems, Logan, Utah) and Hypoxanthine, aminopterin, and thymidine (HAT).
- 5 The cells were distributed into two, 24-well tissue culture trays. Fresh HAT media was added the following day, and twice weekly for two weeks. The cells were cultured an additional two weeks in hypoxanthine- and thymidine-containing MEM and fetal calf serum. Wells supporting the growth of
- 10 hybridomas covering approximately one third the surface area of the well were screened for antibody as described in Example 1 by the enzyme immunoassay procedure.

Generally, at 5 to 8 weeks following the fusion date, cells from positive wells were cloned by limiting

15 dilution. The cells were diluted to 30 cells and 10 cells per ml of media; 0.1 ml of these solutions were dispensed in 96-well trays. An additional 0.1 ml of media containing spleen cells (2.5×10^6 /ml) was added; the trays were wrapped in plastic film and incubated at 37°C in a humidified

20 chamber in the presence of 5% CO₂ for 2 to 6 weeks. The wells were again screened for antibody production. A population was considered cloned when obtained from a well that was one of a group of wells receiving the same cell contractation only if that group had growth of hybridomas in

25 one third or fewer wells. Generally clones were cloned 3 to 4 times to ensure single-cell origin.



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- 1 Positive clones were transferred into 24-well tissue culture plates and after 1 to 3 weeks, into flasks in order to expand the populations. The culture supernatants were collected and stored at 4°C in 0.1% sodium azide.
- 5 Ascites were collected from BALB/c female mice to 4-8 weeks following being primed with pristine (0.5 ml i.p. 10 to 30 days before injection of hybridoma cells) and injected i.p. with 10^6 to 10^7 hybridoma cells.

- B. In a modification of the above procedure,
- 10 P3X63Ag8Z myeloma cells are substituted for the SP2/0 cells and substantially similar results are obtained.



1

EXAMPLE 4Solid Phase Immunometric Assay

The principle of detection of antigens (chlamydial endotoxin or chlamydia in this example) by a solid phase immunometric assay whereby the antigen is sandwiched between two antibodies is well documented (for review see Immunoassays for the 80s, A. Voller, A. Bartlett and D. Bidwell, eds. University Park Press, Baltimore (1981) at page 85. Heterogeneous enzyme immunoassays by S. Avrameas).

10 The general scheme of such an assay is 1) adsorption or covalent linking of the first antibody to a solid phase such as a polystyrene or nylon tube, plate or bead, 2) incubation of the test material containing the antigen which will specifically bind to the first antibody, 3) washing away unreacted antigen(s) not specifically bound, 4) addition of the second antibody which will also react with the antigen already bound to the first antibody. (the second antibody may be labelled by iodination with radioactive iodine, e.g., or by being conjugated with an enzyme such as alkaline phosphatase), 5) washing an unreacted labelled second antibody from the reaction vessel, and finally 6) measuring the presence of the labelled antibody remaining in the vessel (radiometrically or enzymatically).

25 The protocol used herein is after the procedure described by E. Engvall, K. Jonsson, and P. Perlmann (Biochim. Biophys. Acta 251, 427-434 (1971)). In brief the procedure was to coat polystyrene plates with polyclonal or monoclonal antibody to endotoxic glycolipids (see Example 1 and 2 for preparation of these antibodies). The antibody

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1 solutions were diluted in 0.1 M sodium carbonate buffer (pH
10) and 100 ul dispensed into each well of a 96-well
polystyrene plate. They were incubated at 37°C for 3 hours
minimum (maximum of 18 hours), washed with phosphate buffered
5 saline (pH 7.4) containing 0.9% sodium chloride and 0.05%
Tween-20 and 0.5% bovine serum albumin, the test material
containing chlamydia or endotoxic glycolipid antigens diluted
in the same type solution as used for the wash step was added
(100 ul per well), the plates were incubated a minimum of 3
10 hours at 37°C, washed as before (200 ul per well per wash, 4
washes at 3 minute intervals), the second labelled antibody
(immunoglobulin conjugated to alkaline phosphatase by the
procedure described by S. Avrameas (Immunochemistry 6, 43
(1969)) was added and incubated 3-18 hours at 37°C, the
15 reaction vessels were washed as before, 100 ul of substrate
solution containing p-nitro-phenol phosphate (Sigma Chemical
Co., St. Louis, MO) (1 mg/ml) in 1% diethylamine and 1 mM
MgCl₂ was added. The reaction was read visually after
development of a distinct yellow color in positive reaction
20 wells or quantified spectrophotometrically at 405 nm in a
Beckman DU-8 spectrophotometer.



1

EXAMPLE 5Antibody Reactivity in Enzyme Immunoassay

Protocol: The various antigens were coated on the surfaces of polystyrene plates as described in Example 1. The
5 antisera and monoclonal antibody solutions were added and processed as described. The reactivity of these antibodies to the antigens were detected using alkaline phosphatase conjugated to anti-mouse or anti-rabbit immunoglobulins.



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TABLE I

	Source of Antibody	Antigen coated on wells							
		Not treated						Periodate treated	
		None	LA	Re	GL	N.g.	C.t.	N.g.	C.t.
	A. Polyclonal								
	Rabbit anti-Re GL	-	-	++		±	++	-	±
	Rabbit anti-LA	-	++	±		±	+	+	+
10	Mouse anti-Re-LA GL	-	++	++		±	++	+	++
	Mouse anti-C. trachomatis	-	-	-		-	++	-	+
	Mouse anti-N. gonorrhoeae	-	-	-		++	-	+	-
15	Normal Rabbit sera	-	-	-		-	-	-	-
	Normal Mouse sera	-	-	-		-	-	-	-
	B: Monoclonal (mouse)								
	Anti-Re GL (clone:								
	Re-1)-	-	-	++		±	++	-	-
20	Re-2)-	-	-	++		±	++	-	-
	" Re-3)-	-	-	++		±	++	-	-
	Anti-LA (clone:LA-1)-	++	±			-	-	-	+
	LA-2)-	++	±			+	+	-	++
	LA-3)-	++	±			-	±	-	+
25	LA-4)-	++	+			+	+	++	++
	LA-5)-	++	+			+	+	++	++
	Anti-C-GL(clone:C-1)-	-	-			-	++	-	-
	C-2 -	-	-			-	++	-	-
	C-3 -	-	-			-	++	-	-
30	C-4 -	-	-			-	++	-	-
	C-5 -	-	-			-	++	-	-



1 Abbreviations:

- 1) N.g. = Neisseria gonorrhoeae whole cells
2) C.t. = Chlamydia trachomatis elementary bodies
3) LA = Lipid A
5 4) Re GL= Re endotoxic glycolipid
5) C-Gl = Chlamydia glycolipid
" -" (negative) = Absorbancy at 405nm below 0.1
6) "+" (positive) = " between 0.31 & 0.5
"++" (") = greater than 0.5
10 "±" (weakly positive) = between 0.1 & 0.30

From the above data it can be seen that both polyclonal and monoclonal antisera can be generated that will react with endotoxic glycolipids from a variety of sources.
15 Both anti-Re glycolipid antisera and anti-Lipid A antisera reacts with periodate-sensitive determinates in C. trachomatis elementary bodies. Weak reactivity of these antisera is seen to N. gonorrhoeae. The periodate-sensitivity of certain of these reactions is
20 evidence that the determinate recognized by these antisera is the endotoxic glycolipid present in the cell walls of the microbes. The antisera are defined by their reactivity to purified endotoxic glycolipid and Lipid A from Salmonella whole cells. However, these antisera contain antibodies to
25 to other non-endotoxic molecules in the whole cells such as proteins. Only the anti-endotoxic glycolipid antibodies bind to the chlamydial and gonococcal microbes and this binding is in certain cases prevented by pretreating the microbes with periodate which destroys the unprotected sugars of the
30 endotoxic glycolipids within the whole cells or elementary bodies. However, if the epitope comprises a chemically modified sugar (e. g. amino sugars) the periodate treatment is ineffective.



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1 Monoclonal antibodies with reactivity similar to
the polyclonal antisera can also be generated. This
reactivity, too, in certain cases is periodate-sensitive,
thereby providing further evidence that the epitopes being
5 recognized on the C. trachomatis and N. gonorrhoeae are the
sugar moieties of the endotoxic glycolipids in the cell walls
or membranes. In addition, these moieties are
immunologically identical as defined by monoclonal antibody
reactivity to some of the sugar moieties in the endotoxic
10 glycolipids of the core or Lipid A region of gram negative
bacteria.

Certain of the clones described in Table I were
deposited with the American Type Culbun Collection 12301
Parklawn Drive, Rockville, Maryland on January 19, 1984 and
15 the requisite fees were paid. Access to the cultures will be
available during the pendency of the patent application to
one determined by the Commissioner to be entitled thereto
under 27 CFR §1.14 and 35 USC §122. All restrictions on
availability of said culture to the public will be
20 irrevocably removed upon the granting of the instant
application and said culture will remain permanently
available during the term of said patent. Should the culture
become nonviable or be inadvertently destroyed, it will be
replaced with viable culture(s) of the same taxonomic
25 description.



- 1 Each deposited clone was serotyped and assigned the accession number as shown in Table II.

TABLE II

5	<u>Clone</u>		<u>Antibody Type</u>	<u>Accession No.</u>
	Anti-Re GL	Clone Re-1	IgG ₃ (K)	HB8482
	Anti-Re GL	Clone Re-2	IgG ₃ (K)	HB8481
10	Anti-LA	Clone LA-4	IgM (K)	HB8480
	Anti-LA	Clone LA-5	IgM (K)	HB8479
	Anti-C GL	Clone C-2	IgG _{2a} (K)	HB8478



1 WHAT IS CLAIMED IS:

1. An immunological reagent for the detection of shared antigenic determinants of at least two microorganisms comprising an antibody specifically reactive to said shared
5 determinants.

2. The reagent according to Claim 1 wherein said microorganisms are selected from the group consisting of Escherichia, Salmonella, Brucella, Neisseria, Chlamydia, and Rickettsia.

10 3. The reagent according to Claim 1 wherein said determinant is a portion of a macromolecule.

4. The reagent according to Claim 3 wherein said macromolecule is an endotoxic or endotoxic-like lipopolysaccharide.

15 5. The reagent according to Claim 3 wherein said portion of a macromolecule comprises Lipid A region or core oligosaccharide region of an endotoxic or endotoxic-like lipopolysaccharide.

20 6. The reagent according to Claim 5 wherein said region is the Lipid A region.

7. The reagent according to Claim 5 wherein said region is the core oligosaccharide region.

8. The reagent according to Claim 1 wherein said antibody is a monoclonal antibody.

25 9. An immunological reagent for the detection of two or more endotoxin or endotoxin-like lipopolysaccharide producing microorganisms comprising a monoclonal antibody specifically reactive with shared antigenic determinants of said lipopolysaccharide.



1 10. The reagent according to Claim 9 wherein said
microorganisms are selected from the group consisting of
Escherichia, Salmonella, Brucella, Neisseria, Chlamydia, and
Rickettsia.

5 11. The reagent according to Claim 9 wherein said
determinants comprises the Lipid A region or core
oligosaccharide region of said lipopolysaccharide.

 12. The reagent according to Claim 11 wherein said
determinant comprises the Lipid A region.

10 13. A method for the immunological detection of an
antigen shared by a plurality of taxonomically distinct
microorganisms comprising:

 contacting a sample containing said microorganisms
or antigens thereof with an analytically indicatable antibody
15 to form an immunocomplex of said microorganism or antigen
thereof and said antibody, said antibody being characterized
as having affinity for an antigenic determinant shared by
said microorganism.

 14. The method according to Claim 13 wherein said
20 antibody is analytically detected by fluorometric, enzymatic
or radiometric measurement of fluorescent, enzymatic or
radioactive material, chemically complexed with said
antibody.

 15. The method according to Claim 13 wherein said
25 indicatable antibody is analytically indicated by reaction
with a second antibody specific for said indicatable
antibody, said reaction being indicated by fluorometric,
enzymatic or radiometric measurement of fluorescent,
enzymatic or radioactive material chemically complexed to
30 either of said antibodies.



1 16. The method according to Claim 13 wherein said
shared antigenic determinant is a portion of a cell-surface
macromolecule.

 17. The method according to Claim 16 wherein said
5 macromolecule is an endotoxic or endotoxin-like
lipopolysaccharide.

 18. The method according to Claim 16 wherein
said-portion of a macromolecule comprises a Lipid A region or
core-oligosaccharide region of an endotoxin or endotoxin-like
10 lipopolysaccharide.

 19. The method according to Claim 18 wherein said
region is the Lipid A region.

 20. The method according to Claim 18 wherein said
region is the core-oligosaccharide region.

15 21. The method according to Claim 13 wherein said
sample is a clinical sample selected from the group
consisting of blood, serum, saliva, stool, urine, milk,
topical washings of skin or genitals, tissue samples or
homogenates thereof and samples of cell culture fluids of
20 infected cells or homogenates thereof.

 22. The method according to Claim 13 wherein said
indicatable antibody is a monoclonal antibody.

 23. In a two-site immunometric assay to identify
an antigen in a sample comprising forming a ternary complex
25 of a first labelled antibody, said antigen, and a second
antibody said second antibody being bound to a solid carrier
insoluble under ternary complex forming conditions wherein
the presence of the antigen is detected and identified by
measuring either the amount of labelled antibody bound to
30 said solid carrier or the amount of unreacted labelled
antibody: the improvement comprising employing as said



1 second antibody a monoclonal antibody characterized in having
affinity for an antigenic determinant site of said antigen
which is shared by a plurality of taxonomically distinct
microorganisms and employing as a first antibody an antibody
5 characterized as having an affinity for an antigenic
determinant site specific to a taxonomically distinct
microorganism.

24. The method according to Claim 23 wherein said
antigenic determinant site reactive with said first antibody
10 and said antigenic determinant site reaction with said second
antibody are regions of the same macromolecule.

25. The method according to Claim 24 wherein said
macromolecule is an endotoxic or endotoxic-like
lipopolysaccharide.

15 26. The method according to Claim 24 wherein said
antigenic determinant reaction with said second antibody is
comprised of the Lipid A region or core oligosaccharide
region of an endotoxic or endotoxic-like lipopolysaccharide.

27. The method according to Claim 26 wherein said
20 region is the Lipid-A region.

28. The method according to Claim 26 wherein said
region is the core oligosaccharide.

29. The method according to Claim 24 wherein said
antigenic determinant site reaction with said first antibody
25 is comprised of the somatic region of an endotoxic or
endotoxic-like lipopolysaccharide.

30. The method according to Claim 24 wherein said
antigenic determinant site reactive with said second antibody
and said antigenic determinant site reactive with said first
30 antibody are regions of separate macromolecules.



1 31. The method according to Claim 30 wherein said
antigenic determinant site reactive with said second antibody
is the Lipid A or core oligosaccharide region of an endotoxic
or endotoxic-like lipopolysaccharide.

5 32. The method according to Claim 30 wherein said
antigenic determinant site reactive with said first antibody
is a region of any nonendotoxic or endotoxic-like
lipopolysaccharide molecule with the proviso that said
determinant is specific to a taxonomically distinct
10 microorganism.

33. The method according to Claim 24 wherein said
first labelled antibody is labelled with a fluorometric,
enzymatic or radioactive material.

15 34. A monoclonal antibody produced by a hybridoma
formed by a fusion of cells from a mouse myeloma and spleen
cells from a mouse previously immunized with Salmonella Re
endotoxin glycolipid.

35. The monoclonal antibody of Claim 34 which is
produced from a hybridoma formed by the fusion of SP2/0
20 myeloma cells and Balb/c mouse spleen cells.

36. The monoclonal antibody of Claim 34 wherein
said hybridoma is selected from the group consisting of Clone
Re-1 (ATCC. No. HB 8482) and Re-2 (ATCC. No. 8481).

25 37. A monoclonal antibody produced by a hybridoma,
formed by a fusion of cells from a mouse myeloma and spleen
cells from a mouse previously immunized with Lipid A.

38. The monoclonal antibody of Claim 37 which is
produced from a hybridoma formed by the fusion of SP2/0
myeloma cells and Balb/c mouse spleen cells.

30 39. The monoclonal antibody of Claim 37 wherein
said hybridoma is selected from the group consisting of Clone
LA-4 (Ascc. No. HB8480) and LA-5 (Ascc. No. HB8479).



1 40. A monoclonal antibody produced by a hybridoma
formed the fusion of cells from a mouse myeloma and spleen
cells from a mouse previously immunized with Chylamvdia
glycolipid.

5 41. The monoclonal antibody of Claim 40 which is
produced from a hybridoma formed by the fusion of SP2/0
myeloma cells and Balb/c mouse spleen cells.

 42. The monoclonal antibody of Claim 40 wherein
said hybridoma is Clone C-2 (ATCC. No. HB 8478).

10 43. A kit for use in the immunological detection
of an antigen shared by a plurality of taxonomically distinct
microorganisms comprising antigen reactive means comprising
an antibody specifically reactive with said antigen, said
antibody being further characterized in being chemically
15 complexed with an analytically detectable reagent.

 44. The kit of Claim 43 wherein said reagent
comprises fluoroscent, enzymic or radioactive material.

 45. The kit of Claim 44 wherein said reagent is
the enzyme alkaline phosphatase.

20 46. The kit of Claim 43 wherein said shared
antigen is an endotoxic or endotoxic-like lipopolysaccharide.

 47. The kit of Claim 43 wherein said antibody is
the monoclonal antibody of Claim 34.

25 48. The kit of Claim 43 where said antibody is the
monoclonal antibody of Claim 37.

 49. The kit of Claim 43 wherein said antibody is
the monoclonal antibody of Claim 40.

30 50. A kit for use in the immunological detection
of two or more endotoxin or endotoxin-like lipopolysaccharide
producing microorganisms comprising:



- 1 1) a lipopolysaccharide reactive means comprising
a first antibody specifically reactive with
said lipopolysaccharide, and
- 5 2) a second antibody specifically reactive with
said first antibody, said second antibody
being further characterized in having
chemically complexed thereto an analytically
detectable reagent.
- 10 51. The kit of Claim 50 wherein said reagent
comprises fluoroscent, enzymic, or radioactive material.
52. The kit of Claim 51 wherein said reagent is
the enzymic alkaline phosphatase.
53. The kit of Claim 50 wherein said first
antibody is the monoclonal antibody of Claim 34.
- 15 54. The kit of Claim 50 wherein said first
antibody is the monoclonal antibody of Claim 37.
55. The kit of Claim 50 wherein said first
antibody is the monoclonal antibody of Claim 40.
- 20 56. A kit for use in a two-site immunometric assay
for the identification of a plurality of taxonomically
distinct lipopolysaccharide producing microorganisms
comprising:
- 25 1) a first lipopolysaccharide reactive means
comprising a first antibody specifically
reactive with an antigenic determinant of said
lipopolysaccharide said determinant being
specific to a taxonomically distinct
microorganism, said first antibody being
further characterized in having chemically
30 complexed thereto an analytically detectable
reagent, and



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- 1 2) a second lipopolysaccharide detecting means
 comprising a second antibody specifically
 reactive to an antigenic determinant of said
 lipopolysaccharide, said determinant being
5 shared by a plurality of lipopolysaccharide
 producing microorganisms said second antibody
 being further characterized in being bound to a
 solid carrier.

57. The kit of Claim 56 wherein said reagent
10 comprises fluoroscent, enzymic, or radioactive material.

58. The kit of Claim 57 wherein said reagent is the
the enzyme alkaline phosphatase.

59. The kit of Claim 56 wherein said microorganisms
are selected from the group consisting of Escherichia,
15 Salmonella, Brucella, Neisseria, Chlamydia, and Rickettsia.

60. The kit of Claim 56 wherein said second
antibody is the monoclonal antibody of Claim 34.

61. The kit of Claim 56 wherein said second
antibody is the monoclonal antibody of Claim 37.

20 62. The kit of Claim 56 wherein said second
antibody is the monoclonal antibody of Claim 40.

63. A murine hybridoma cell line capable of
producing a monoclonal antibody which is immunologically
reactive iwth shared determinants of an endotoxin or
25 endotoxin-like lipopolysaccharide.

64. The cell line according to Claim 63 selected
from the group consisting of cell line Re-1 (ATCC. No. HB
8482), Re-2 (ATCC. No. HB8481), LA-4 (ATCC. No. HB 8480),
LA-5 (ATCC. No. HB 8479) and C-2 (ATCC. No. HB 8478).

30



INTERNATIONAL SEARCH REPORT

International Application No PCT/US84/02022

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
US - 435/7, 810, 948; 260/112B IPC - G01N 33/54; A61K 39/395		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
US	435/7, 18, 21, 34, 35, 39, 68, 172.2, 240, 810, 948 436/548,511, 807 260/112B, 112R 935/95,102, 103, 106, 110	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
MEDLINE 1966-1984 Biosis Previews -1969-1985 Chemical Abstracts -1967-1984		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	N, Science, <u>220</u> , issued 17 June 1983, Nurminen et al, 1279-1281.	1-5, 7, 13- 18,20, 21, 43- 46, 50-52
X	N, Journal of Immunological Methods, <u>43</u> , issued 1981, Stevens et al, 199-207	1-5, 7, 13, 16-18, 20, 43, 44, 46
X	N, Infection and Immunity, <u>34(3)</u> , issued December 1981, Apicella et al, 751-756	1-5, 7-11, 13-18, 20-22, 43-46,50-52, 63
X	N, European Journal of Immunology, <u>12</u> , issued 1982, Hiernaux et al. 797-803	1-5, 7-11, 13-18,20-22, 43-46, 50-52, 63
X	N, The Journal of Immunology, <u>115(4)</u> , issued October 1975, Caldwell et al, 963-968	1-5
<p>* Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"d" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹	Date of Mailing of this International Search Report ²	
14 January 1985	18 JAN 1985	
International Searching Authority ¹	Signature of Authorized Officer ¹⁹	
ISA/US	ESTHER M. KEPPLINGER	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	N, Science, 220, issued 17 June 1983, Nurminen et al, 1279-1281	8-12, 19, 22-42, 47-49, 53-64
Y	N, Journal of Immunological Methods, 43, issued 1981, Stevens et al, 199-207	19, 22-39, 47, 48, 53, 54, 56-61, 63, 64
Y	N, European Journal of Immunology, 12, issued 1982, Hiernaux et al, 797-803	19, 22-39, 47, 48, 53, 54, 56-61, 63, 64
Y	N, The Journal of Immunology, 115(4), issued October 1975, Caldwell et al, 963-968	40-42, 49, 55, 62-64
Y,P	N, The Journal of Infectious Diseases, 149(4), issued April 1984, Puolakkainen et al, 598-604	1-64
Y,P	N, Infection And Immunity, 44(2), issued May 1984, Caldwell et al, 306-314	1-64
Y,	N, Infection And Immunity, 45(3), issued September 1984, Mutharia et al, 631-636	1-64

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	N, Infection and Immunity, 42(3), issued December 1983, Sugawara et al, 863-868	1, 3-5, 8, 9, 11, 13-18, 21, 22, 43, 44, 46, 50, 51, 63
X	US, A. 4,115,543, Published 19 September 1978 See Column 4, lines 11-13, Wallace	1, 3, 4, 13
Y	US, A. 4,376,110, Published 08 March 1983, David	1-64

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.